Anchoring of Vascular Endothelial Growth Factor to Surface-Immobilized Heparin on Pancreatic Islets: Implications for Stimulating Islet Angiogenesis

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In pancreatic islet transplantation, early revascularization is necessary for long-term graft function. We have shown in *in vitro* and *in vivo* models that modification with surface-attached heparin protects the islets from acute attack by the innate immune system of the blood following intraportal islet transplantation. In this study, we have investigated the ability of an immobilized conjugate composed of heparin to bind the angiogenic growth factor vascular endothelial growth factor-A (VEGF-A) as a means of attracting endothelial cells (ECs) to induce angiogenesis and revascularization. We analyzed the capacity of VEGF-A to bind to immobilized heparin and how this affected the proliferation and adherence of ECs to both artificial glass surfaces and islets. Quartz crystal microbalance with dissipation monitoring and slot-blot demonstrated the binding of VEGF-A to heparin-coated surfaces upon which ECs showed protein-dependent proliferation. Also, ECs cultured on heparin-coated glass surfaces exhibited effects upon focal contacts. Heparinized islets combined with VEGF-A demonstrated unaffected insulin release. Further, covering islets with heparin also increased the adhesion of ECs to the islet surface. Immobilized heparin on the islet surface may be a useful anchor molecule for achieving complete coverage of islets with angiogenic growth factors, ultimately improving islet revascularization and engraftment in pancreatic islet transplantation.

Introduction

CLINICAL ISLET TRANSPLANTATION is emerging as an established procedure for treatment of patients with type-1 diabetes. However, in most patients, islets from more than one donor are needed to achieve insulin independence, indicating that only a small fraction of the transplanted islets successfully engraft in the liver after infusion into the portal vein.^{1–3} A number of studies have demonstrated that the reestablishment of an appropriate microvascular supply is an essential prerequisite for successful islet engraftment.^{4–6}

Growth factors, particularly vascular endothelial growth factor (VEGF), are known to contribute significantly to the vascularization of transplanted islets.^{7–11} The VEGF family of homodimeric glycoproteins in humans consists of VEGF-A, -B, -C, -D and placental growth factor. VEGF-A is critical during development as shown by lethality of transgenic mice lacking one allele.¹² In hypoxia-driven processes such as angiogenesis, the formation of new blood vessels by sprouting from preexisting vessels,¹³ hypoxia-regulated VEGF-A mRNA transcription is increased.^{14,15} VEGF-A stimulates endothelial cell (EC) permeability and chemotaxis through cognate VEGF receptors (VEGFRs), where VEGFR-2

is the major mediator of the effects of VEGF-A.^{16,17} VEGF-A is continuously expressed in normal pancreatic islets^{18–20} and at particularly high levels in devascularized and hypoxic pancreatic islets.^{21,22} Further, underscoring its role in islet biology is the observation that animals lacking specific islet VEGF-A expression in pancreatic islets have continuous, instead of fenestrated capillaries.^{20,23} The locally expressed VEGF-A is a prerequisite for islet endothelial fenestration, as has been previously shown for other tissues.^{24,25}

We have recently demonstrated in *in vitro* and *in vivo* models that modification of pancreatic islets with surface-attached heparin conjugates, consisting of approximately 70 heparin molecules covalently attached to a carrier backbone,²⁶ can protect the islets from acute attack by the innate immune system of the blood after intraportal islet cell transplantation.²⁷ The application of immobilized heparin directly to the islet surface mimics the protective biological activity exerted by heparan sulfate proteoglycans (HSPGs) at the endothelial lining of the vascular wall and thereby provides protection against innate immune reactions.

Another potentially advantageous feature of heparin in this setting is its capacity to bind angiogenic growth factors, including VEGF-A,¹⁶ through the heparin-binding domains.

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VEGFR-2 is expressed on ECs^{28,29} and binding of VEGF-A creates dimerization of the receptors leading to activation of intrinsic receptor tyrosine kinase activity.³⁰ The tyrosine kinases stimulate phosphorylation cascades of intracellular proteins which finally lead to effects such as survival, proliferation, and migration of ECs. To become stabilized, the receptors and growth factors must interact with glucosaminoglycans such as HSPGs, which are expressed on the cell surface of the ECs and neighboring cells. HSPGs also act as a reservoir of growth factors on the cell surface.^{31,32} Heparin, which is a structurally related but more heavily sulfated glucosaminoglycan, can mimic many of the features of the HSPGs.^{33,34} Indeed, heparin conjugates anchored onto the islet surface may well trigger revascularization processes. A first step in testing this possibility is to investigate the ability of heparin conjugates to bind VEGF-A and attract ECs, thereby inducing angiogenesis and revascularization.

In this study, we have examined the capacity of immobilized heparin conjugate to bind VEGF-A and have assessed its effects upon ECs. We have demonstrated by a variety of techniques that modification of surfaces with immobilized heparin increase the binding of ECs and their proliferation after VEGF stimulation, when compared with results obtained with unmodified surfaces. These results have important implications for improving the survival and function of human pancreatic islets after transplantation.

Materials and Methods

Islet isolation

Human pancreases were obtained within the Nordic Network from diseased donors after appropriate consent for multiorgan donation. The islets were isolated at the Division of Clinical Immunology at the University of Uppsala, using a modification of a previously described semiautomated digestion–filtration method.^{35–37} The purity of islet preparations used in this study ranged from 70% to 95% and 3–14 donors per experiment were used.

Culture of ECs

Human dermal microvascular ECs (PromoCell GmbH, Heidelberg, Germany) were cultured using EC growth medium MV with supplement mix (ECGM MV; PromoCell GmbH). The EC used were from passage 3 to 12.

Islet and surface heparinization procedure

Human islets were biotinylated by incubating the islets for 30 min at room temperature (RT) in Connaught Medical Research Laboratories (CMRL; Cellgro, Mediatech, Inc., Manassas, VA) culture medium without serum but containing 1 mg/mL EZ-Link™ Sulfo-NHS-LC-biotin (Pierce Biotechnology, Rockford, IL). The islets were washed twice and then incubated for 30 min at 37°C in culture medium supplemented with 1 mg/mL of avidin (Pierce Biotechnology). Finally, macromolecular conjugates of heparin (Corline Systems AB, Uppsala, Sweden) at 1 mg/mL in culture medium were allowed to bind to the biotin/avidin coating for 60 min.

All plates (24-well) used for the thymidine assay and cover glass surfaces used for culture of ECs were preincubated with 2% bovine serum albumin (BSA) or 6 µg/mL collagen

PureCol (INAMED Biomaterials/Nutacon BV, Leimuiden, The Netherlands) in phosphate-buffered saline (PBS) and thereafter heparinized as described earlier. Sulfated tissue culture plastic was used as control surface, which was prepared by adding 0.2 g/mL KMnO₄ dissolved in concentrated H₂SO₄ and incubated during 2 min of continuous swirling.

Quartz crystal microbalance with dissipation monitoring of VEGF-A binding

To monitor the capacity of VEGF-A to bind to immobilized heparin, a quartz crystal microbalance with dissipation monitoring (QCM-D) approach (Q-Sense AB, Gothenburg, Sweden) was used.³⁸ This technique relies on the fact that a mass adsorbed onto the sensor surface of a shear-mode oscillating quartz crystal causes a proportional change in its resonance frequency, f . Changes in f reflect the amount of mass deposited onto the surface of the crystal. The corresponding dissipation change, ΔD , indicates frictional (viscous) losses induced by the deposited materials. Sensor crystals (5-MHz) sputtered with stainless steel were used. The crystals were incubated in 2% human albumin (Baxter AG, Vienna, Austria) for 30 min at RT, and heparinization was carried out as described earlier. The final step included binding of human VEGF-A (6.7 µg/mL; PeproTech, London, UK).

Slot-blot assay of VEGF-A binding to heparinized surfaces

VEGF-A (PeproTech) was diluted in Tris-buffered saline (TBS) with 0.1% BSA in the concentrations of 1.5, 3.1, 6.2, 12.5, 25, and 50 ng/mL. A volume of 100 µL VEGF-A in the specified concentrations was added in duplicates to heparinized microtiter wells. VEGF-A solutions were incubated on the heparinized surface for 60 min at RT. Subsequently, the supernatants were collected and the duplicates were pooled into slots of a 48-well slot-blot sandwich dish (Bio-Rad, Hercules, CA) in which the solutions were bound to a nitrocellulose membrane through vacuum pressure. Samples of 1.5–50 ng/mL VEGF-A in 0.1% BSA/TBS were used as comparative controls (100 µL collected in duplicates) in the slot blots. The nitrocellulose membrane was then blocked in 5% dry milk dissolved in TBS with 0.05% Tween-20 (TBS-T). Immunoblotting was performed by rabbit anti-human VEGF-A antibody (product no. AF-293-NA; R&D Systems, Minneapolis, MN) diluted 1:100 in 5% dry milk in TBS-T and incubated overnight in 4°C. After vigorous washes of the membrane in TBS-T, secondary goat anti-rabbit horse radish peroxidase-conjugated antibody (product no. A4174; Sigma-Aldrich, St. Louis, MO) diluted 1:2500 in 5% dry milk in TBS-T was incubated for 1 h at RT, followed by vigorous washes in TBS-T. Immunoreactive bands were then visualized using enhanced chemiluminescence (ECL) (Immobilon Western Chemilum horse radish peroxidase substrate; Millipore, Billerica, MA). Quantification of blots was performed using Image Gauge ver. 3.3 (FUJI Film Photo, Tokyo, Japan).

Thymidine incorporation assay of EC proliferation

Cell culture plastic was heparinized as described earlier and ECs were analyzed upon interaction with the surface upon seeding and during culture over time.

FIG. 1. Quartz crystal microbalance with dissipation monitoring analysis of growth factor binding to an immobilized heparin conjugate. The polystyrene surface, used as a proxy for the islet surface, was initially coated with albumin to create a matrix to which biotin could bind. Biotin binding was followed by sequential addition of avidin, heparin conjugate, and finally vascular endothelial growth factor-A (VEGF-A). For each step at which an increased mass (= binding) was added to the sensor, a corresponding dampening of the frequency was obtained (f , blue line). The corresponding dissipation change, ΔD , is indicated by purple line. Binding of VEGF-A to the final heparin surface produced a distinct change in both Δf and ΔD .

FIG. 2. Binding of VEGF-A to heparinized surfaces and analyses of proliferation. (A) The amount of added VEGF-A (0.1–5 ng) remaining in solution after incubation on heparinized surfaces (heparin) or in control tubes (control) was assessed by slot-blot. (B) Quantification of VEGF-A in slot-blot. (C) Proliferation by thymidine incorporation (counts per minute) of endothelial cell (EC) seeded in starvation medium (basal) onto heparinized surfaces (bovine serum albumin [BSA] or collagen protein base) preincubated with VEGF-A (VEGFpre) or in the presence of soluble VEGF-A (VEGFs, 1 ng/mL); $n = 4$, $*p < 0.05$, t -test. The relevant mean counts per minute values of four individual experiments were BSA–heparin basal, 476 ± 83 ; BSA–heparin VEGFpre, 975 ± 208 ; collagen–heparin basal, 1469 ± 205 ; collagen–heparin VEGFpre, 2642 ± 274 ; or sulfate basal, 785 ± 90 ; sulfate VEGFpre, 924 ± 157 . (D) ECs cultured over time before stimulation with added VEGF-A after 24 h of starvation. Sulfated surfaces were used as control surface with negatively charged sulfate groups.

Heparinized surfaces and control sulfated surfaces were preincubated with VEGF-A (10 ng/24-well; PeproTech) for 1 h at RT, followed by two washes with TBS to remove unanchored VEGF-A. Surfaces without added growth factor were denoted "basal." The ECs were then plated at 2.5×10^4 cells/well in 1 mL of ECGM MV with 0.5% fetal bovine serum (starvation medium) and cultured for 16 h at 37°C before thymidine incorporation (see below). Soluble VEGF-A (1 ng/mL) was added as indicated upon seeding of cells.

Also, ECs were plated at 2.5×10^4 cells/well in 1 mL of ECGM MV with supplements. After 24 h of culture, the cells were washed once and incubated in starvation medium for another 24 h at 37°C. Cells were stimulated with or without human VEGF-A (20 ng/mL; PeproTech) as indicated and incubated for 16 h at 37°C.

After the 16-h incubation, [^3H] thymidine (1 $\mu\text{Ci/mL}$) incorporation was performed for a 4-h incubation period at 37°C. The cells were placed on ice and washed in ice-cold PBS followed by incubation in ice-cold 10% trichloro acetic acid for 20 min and then washed twice in ice-cold 95% ethanol. The precipitated DNA was solubilized in 0.2 M NaOH and the contents of the wells were transferred to scintillation vials; [^3H] thymidine incorporation was determined by liquid scintillation counting. Samples were analyzed in triplicate. Representative results are shown with statistics based on four individual experiments.

ECs cultured on heparinized surface

Pieces of cover glass were precoated with 6 $\mu\text{g/mL}$ collagen PureCol (INAMED Biomaterials/Nutacon BV) and then heparinized as described earlier or left untreated. Glasses were incubated for 1 h with VEGF-A (20 ng/mL; PeproTech) and then washed twice with TBS. The cover glasses were placed in a 24-well plate, and 25,000 ECs/well were seeded in ECGM MV with 0.5% FBS. After 24 h, the cells were washed once in ice-cold TBS and fixed in zinc fix (Becton Dickinson, Franklin Lakes, NJ) for 30 min. Staining of focal adhesions was performed by mouse anti-paxillin antibody (product no. 610052; Becton Dickinson) diluted 1:500, followed by washes in TBS and incubation with Alexa 488-labeled goat anti-mouse highly cross-adsorbed IgG (product no. A-11029; Molecular Probes, Eugene, OR) diluted 1:1000. Staining of cytoskeletal actin was performed by Texas red-conjugated phalloidin antibody (product no. T7471; Molecular Probes) diluted 1:200. Nuclei were stained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich). Cover glasses were mounted with Fluoromount-G mounting medium (Southern Biotechnology, Birmingham, AL). The results were analyzed by confocal laser scanning microscopy (Zeiss LSM 510 Meta; Carl Zeiss AG, Göttingen, Germany).

Confocal microscopy analysis of human islets after incubation with VEGF-A

The binding of VEGF-A to the surface of heparinized or untreated control islets was analyzed after incubating the islets at RT for 60 min with human VEGF-A (2 ng/mL, PeproTech) fluorescently labeled using Alexa Fluor 488 Protein Labelling Kit (Molecular Probes). Images were acquired by confocal laser scanning microscopy (Zeiss LSM 510 Meta; Carl Zeiss AG).

Insulin release analysis by dynamic perfusion system

Insulin secretion in response to glucose stimulation in a dynamic perfusion system was assessed as previously described³⁹ at 24 h after heparinization. Islets were initially perfused with 1.67 mmol/L glucose, thereafter with 16.7 mmol/L, and finally again with 1.67 mmol/L glucose. During the 120-min perfusion, fractions were collected at 6-min intervals. The concentration of insulin was analyzed using a commercial ELISA kit (Mercodia, Uppsala, Sweden).

Cell labeling

ECs were labeled with Cell Tracker™ Orange CMRA (CT Orange; Molecular Probes) according to the manufacturer's protocol. The cells were incubated at 37°C for 60 min and then washed two times in PBS to remove excess dye.

EC coating of islets of Langerhans

Heparinized or unmodified control human islets were prepared as described earlier. After the heparinization process, the islets (heparinized and control islets) were also incubated with 20 ng/mL of VEGF-A in CMRL medium without serum for 1 h at RT, followed by two washes with CMRL medium. Thereafter the islets were mixed together with 1.2×10^5 CT Orange-labeled EC in 500 μL ECGM MV culture medium according to a previously established method.⁴⁰ The EC-coated islets were transferred to petri dishes, treated to prevent cellular adherence, and cultured for 24–48 h in ECGM MV culture medium. The EC coating of islets was examined using confocal laser scanning microscopy (Zeiss LSM 510 Meta). The relative value of the surface coating between untreated and treated islets as indicated was quantified using the Leica QW software (Leica, Wetzlar, Germany).

Statistical analysis

Statistical analysis was performed using Prism 5.0 (Graph Pad, La Jolla, CA). Data are presented as means $\pm$ standard error of the mean. Mean values were compared using a two-tailed Wilcoxon test or two-tailed *t*-test for paired data. Significance was set at $p \leq 0.05$.

Results

QCM-D analysis of the binding of VEGF-A to heparin surface

Figure 1 shows the sequential binding of biotin, avidin, heparin complexes, and VEGF-A as analyzed by QCM-D. The mass adsorbed onto the oscillating sensor surface causes a proportional change in its resonance frequency, f (blue line). The corresponding dissipation change, ΔD , is indicated by the purple line. The changes in frequency reflect the amount of mass deposited onto the sensor surface. Because of its low molecular weight, binding of biotin resulted in a low frequency shift, followed by a rapid frequency increase reflecting the buffer exchange. Binding of avidin to biotin was found to occur with an obvious change in Δ but no change in ΔD , indicating a rigid binding, whereas binding of the heparin conjugates to avidin resulted in a distinct change in both

FIG. 3. Morphology analyses of ECs cultured on heparinized and untreated surfaces. (A) Induced adherence of ECs toward heparin-coated surface was shown at 6 h after seeding of cells compared with control. The formation of stress fibers and focal adhesions was assessed in ECs after 24 h of culture on untreated glass surfaces or collagen-heparin-coated glass surfaces preincubated with VEGF-A. Stress fibers were visualized with phalloidin (red) and focal adhesions with paxillin (green). Paxillin was localized as dense focal adhesions on control surfaces (B, arrow heads) and along the cytoskeleton in fibrillar adhesions on collagen-heparin surfaces (C, asterisks). Nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Scale bars = 20 μ m. The pictures to the far right in (B) and (C) are high-magnification images of the focal and fibrillar adhesions, respectively.

ΔD and Δf , indicating a more loose and flexible structure. Binding of VEGF-A to the final heparin surface was shown to occur with a distinct change in both Δf and ΔD .

Binding of VEGF-A to heparinized surfaces as analyzed by slot-blot

To investigate the quantitative capacity of the heparinized surface to bind VEGF-A, increasing amount of VEGF-A (1.5–50 ng/mL) was incubated on the heparinized surfaces. The amount of VEGF-A remaining in the supernatants after 1 h of incubation at RT was analyzed by slot-blot (Fig. 2A). Incubation of VEGF-A on heparinized surface resulted in binding of the growth factor to the heparin conjugate (consumption), reaching a maximum binding at approximately 0.3–0.6 ng/cm². The result was quantified by image analysis showing 100% loss of VEGF-A in the supernatant at the concentration of 0.3 ng/cm² and approximately 40% remaining in the supernatant at 0.6 ng/cm² (Fig. 2B).

Proliferation of ECs cultured on untreated and heparinized surfaces

To investigate the capacity of ECs to respond to VEGF-A anchored to heparinized surfaces, the ECs were seeded in starvation medium in a 24-well plate. The amount of bound VEGF-A to the heparinized surfaces was verified by similar proliferation capacity in comparison to the proliferation of ECs in the presence of soluble VEGF-A (1 ng/mL) added in the culture supernatant upon seeding of ECs (Fig. 2C). The control sulfated surface preincubated with VEGF-A showed unaffected effects upon the seeded ECs in comparison to basal, indicating very low binding of VEGF-A (Fig. 2C). At basal conditions the proliferation of ECs on heparinized surface was reduced when BSA was used as protein surface compared with collagen. The sulfated surface used as a control surface of negatively charged sulfate groups showed only a slightly lower proliferation than the collagen–heparin surface (Fig. 2C). In the experiment where ECs were let to bind to the heparinized surface in the presence of complete EC medium followed by starvation and stimulation, VEGF-A added in solution (20 ng/mL) showed similar capacity to stimulate proliferation in all three conditions but still an affected proliferation was seen in basal medium comparable to the seeding experiment (Fig. 2D).

Morphology analysis of ECs on heparinized surfaces

Morphological analyses of ECs cultured on glass surfaces were performed by staining for stress fibers and focal adhesions. Six hours after seeding, an obvious difference in adherence was seen. ECs cultured on heparinized surfaces spread more rapidly compared with untreated surfaces (Fig. 3A). ECs were also seeded on either control or collagen–heparinized glass slides preincubated with VEGF-A (Fig. 3B, C). After 24 h, it appeared that ECs on the control glass surface (Fig. 3B) showed dense focal adhesions (paxillin; green) in the periphery of the cells in comparison to ECs on heparinized surface (Fig. 3C) where the paxillin-positive proteins formed fibrillar adhesions along the phalloidin-positive (red) cytoskeleton. This phenomenon of fibrillar adhesions was also seen on the heparinized surface in the absence of VEGF-A, but to a lower degree (data not shown). The panels to the far right in Figure 3B and C show

detailed enlargement of the focal and fibrillar adhesions, respectively.

Binding of VEGF-A to the islet surface and functionality analysis of heparinized islets

Confocal microscopy was used to verify the binding of VEGF-A to the surface of human islets. This technique revealed successful binding of VEGF-A to the surface of heparinized islets (Fig. 4B). VEGF-A binding to the surface of control islets could not be detected (Fig. 4A). Heparinized islets in the presence or absence of VEGF-A and untreated control islets from three islet preparations responded similarly to glucose stimulation, demonstrating that the normal biphasic insulin secretion was not affected by the heparinization procedure or VEGF-A treatment (Fig. 4C).

Improved coating of islets with EC after heparinization

Quantification of confocal microscopy analysis revealed that islets with heparin conjugate were associated with a threefold increased adhesion of ECs in comparison to non-heparinized islets (Fig. 5A) after 24 h of culture. The average islet surface area coated by ECs after 24 h was 12.2% $\pm$ 3.4% for control islets and 36.2% $\pm$ 10.9% for heparinized islets. After 2 days of culture, the proportion of coating between control and heparinized islets was maintained and the ECs on heparinized islets were also elongated on the islet surface compared with control (Fig. 5A). EC coverage of heparinized islets preincubated with VEGF-A was further increased approximately 1.5-fold compared with immobilized heparin alone (Fig. 5B); this effect was absent in control islets (Fig. 5C).

Discussion

In this study, we have demonstrated the binding of VEGF-A to immobilized macromolecular conjugates of heparin on artificial surfaces and islets of Langerhans to investigate the effects of binding with respect to promoting angiogenesis. The capacity of VEGF-A to bind the heparinized surface showed in slot-blot an approximate saturation of bound VEGF-A at 0.3–0.6 ng/cm². Our group has previously established an approach for improving islet revascularization by coating islets with ECs.^{40,41} In this study, we questioned whether the EC coating efficiency could be affected by VEGF-A anchored to the surface of heparinized islets. Confocal microscopy of the islets revealed significant increased adhesion of ECs to the surface of heparinized islets, an effect that was further improved when heparinized islets were preincubated with VEGF-A. Preincubation of control islets with VEGF-A created no additional effect upon coating. These results indicate the possibility of anchoring growth factors with affinity for heparin on biological surfaces coated with immobilized heparin. This can be implicated in several areas where a defined surface would be beneficial.

Stimulation of ECs on the heparinized surfaces with VEGF-A was evaluated in two experimental setups: exposure to bound VEGF-A upon seeding of cells or VEGF-A in solution. Anchored VEGF-A to the immobilized heparin could symbolize a scenario where migrating ECs are exposed to surfaces such as heparinized islets or heparinized biomaterials with bound growth factor, whereas VEGF-A in solu-

FIG. 4. Binding of VEGF-A to human islets and analysis of insulin release. Alexa Fluor 488-labeled VEGF-A (green) was incubated with control (A) and heparinized (B) human islets and then examined by confocal microscopy. Nuclei were stained by 4',6-diamidino-2-phenylindole (blue). Scale bars = 100 μ m. (C) Insulin release from control (closed diamonds), heparinized (closed squares), and heparinized islet with VEGF-A (closed triangles) cultured for 24 h ($n = 3$). The islets were sequentially stimulated with 1.67, 16.7, and 1.67 mmol/L of glucose. The mean $\pm$ standard error of the mean is shown.

tion would represent tissue-secreted VEGF-A. The ECs seeded on heparinized surfaces preincubated with VEGF-A showed strong response toward the anchored growth factor. It is interesting to speculate about the accessibility of the growth factor in the presence of heparin as ECs seeded on the sulfated surface failed to respond to VEGF-A in solution. Over time, the cells on sulfated surface could equally well respond to VEGF-A in solution as in comparison to heparinized surfaces. This indicates that the heparinized surface can clearly bind VEGF-A and create a rapid stimulation of ECs upon interaction compared with control surface.

Further, ECs seeded on heparinized surfaces in the presence of anchored VEGF-A showed affected focal contacts. In comparison to control surfaces preincubated with VEGF-A in which the ECs formed dense focal adhesions in the periphery of the cell surfaces, ECs on the heparinized surface with anchored VEGF-A expressed paxillin-positive fibrillar adhesions, dot-like spots, along the cytoskeleton. Fibroblasts

expressing focal adhesions are less prone to migrate in comparison to cells forming fibrillar adhesions.⁴² Migration is tightly linked to proliferation and the formation of fibrillar adhesions on heparinized surfaces even in the absence of VEGF-A fits well with the increased proliferation of ECs seeded on the collagen-heparin surface compared with control. Heparin has also the capacity to affect the three-dimensional structure of proteins such as fibronectin. In recent studies by Mitsi *et al.*,^{43,44} it was shown that the capacity of VEGF to interact with fibronectin was enhanced by heparin through conformational changes in VEGF-binding sites of fibronectin. ECs produce fibronectin,⁴⁵ which might directly interact with the heparinized surface and with anchored VEGF-A.

It was shown that heparinization on a BSA-coated surface affected ECs with reduced proliferation in the absence of VEGF-A, whereas collagen as the protein base for heparinization improved EC proliferation in basal conditions compared

FIG. 5. Coating of pancreatic islets by ECs. **(A)** Quantification of EC coating of islets showed a significant threefold increase in adherence upon immobilized heparin compared with untreated control islets after 24 h of culture ($n = 14$, $**p = 0.008$, Wilcoxon test). Confocal stacking images, combined with bright-field microscopy, after 2 days of culture showed that the coating was maintained and elongated characteristics of ECs on heparin-coated islets were observed compared with control. Scale bars = 100 μm . EC coating was further increased in heparinized islets preincubated with VEGF-A **(B)**, $n = 7$, $*p = 0.03$, Wilcoxon test), an effect absent in control islets precoated with VEGF-A **(C)**, $n = 6-7$, $p = 0.1486$, Wilcoxon test).

with control. The control surface in our study was sulfated plastic surface,⁴⁶ creating negatively charged sulfate groups lacking specificity in growth factor binding. This finding of reduced proliferation on BSA–heparin surface is consistent with a study by Letourneur *et al.*, who demonstrated that immobilized heparin on polysaccharide gel matrices inhibits the growth of EC *in vitro*,⁴⁷ as has previously been shown for immobilized heparin on other matrices.^{48,49} Also, BSA is a more EC inert protein compared with collagen. Further, the matrix-dependent variations in proliferation toward VEGF-A may be due to differential regulation of receptors and proteins involved in EC contact regulation such as VEGFR-2, integrins, and focal adhesion kinase, which are strongly phosphorylated in the presence of VEGF-A.⁵⁰

Several studies have previously demonstrated improved islet graft revascularization in response to the production of recombinant VEGF-A within the islets.^{8,9} However, unlike other pretreatment procedures for introducing VEGF-A to the islets, such as gene therapy,^{8,9,51} anchoring VEGF-A directly to immobilized heparin on the islet surface should not be associated with an increased risk of inducing adverse effects

on islet function^{52,53} or adaptive immune responses. Importantly, islet functionality was not affected by anchoring of VEGF-A in this study because the growth factor in combination with immobilized heparin did not affect insulin release in response to a glucose challenge. Treatment of cultured mouse islets with VEGF-A or fibroblast growth factor-2 has been shown insufficient to rescue the islet vasculature⁵⁴; thus, it appears that simple exposure of cultured islets to growth factors is not sufficient. However, our data support the hypothesis that immobilization of heparin directly on the islet surface might enhance the effect on ECs, because heparin is important for growth factor storage and stabilization of receptor complexes during angiogenesis. The possible rescue effect upon islet vasculature in cultured human islets due to exogenously added VEGF-A remains to be investigated.

Taken together, immobilization of a heparin conjugate decorated with growth factors on islets has a favorable attraction on EC adherence and migration, functions which are important for angiogenic processes after transplantation. The approach presented could improve islet engraftment and survival after implantation, especially at extrahepatic trans-

plantation sites where a rapid supply of nutrients and oxygen is required.

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Disclosure Statement

No competing financial interest exists.

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